In satisfaction of the foregoing objects and advantages, there is provided by the present invention a novel plasmid for insertion in E. coli, containing DNA coding for human preproparathyroid hormone. The plasmid when inserted into E. coli functions to transform the E. coli such that the E. coli then produces multiple copies of the plasmid and thus of the cDNA coding for human preproparathyroid hormone. The plasmid for human insertion into E. coli of the present invention and thus the transformed E. coli are distinguishable over prior art plasmids and microorganisms, for example as described in Hendy et al., supra, in that the plasmid of the present invention contains a double start codon at the 5' end of the DNA coding for preproparathyroid hormone. The presence of the double start codon may cause production microorganisms transformed with a plasmid containing the cDNA to produce preproparathyroid hormone at an increased rate and in an improved yield over prior art transformed microorganisms.

On page 6, delete the second full paragraph, and replace this paragraph with the following in accordance with 37 C.F.R. §1.121. A marked up version showing changes is attached:

Employing the promoter and signal sequence of Staphyloccous aureous protein A we have expressed hPTH in *Escherichia coli* as a secretory peptide. Immunoreactive PTH was isolated both from growth medium and periplasmic space. We obtained up to 10 mg/1 hPTH as judged by reactivity in radioimmunoassay.

On page 7, delete the second full paragraph, and replace this paragraph with the following in accordance with 37 C.F.R. §1.121. A marked up version showing changes is attached:

HPTH immunoreactive material was concentrated from the growth medium by passage through a S Sepharose Fast flow column and eluted quantitatively. Recombinant hPTH was purified by reverse phase HPLC. The column was eluted with a linear gradient of acetonitrille/trifluoroacetic acid. A major peak (fractions 32 and 33) with the same retention time as standard hPTH(1-84) was resolved into two peaks in a second HPLC purification step. The major peak from the 2.HPLC eluted exactly as standard hPTH(1-84) and co-

chromatographed with hPTH(1-84) as one symmetric peak. SDS-PAGE of the peak fraction showed one band co-migrating with hPTH standard suggesting that the recombinant PTH was essentially pure. The recombinant hPTH was subjected to N-terminal amino acid analysis. We were able to determine unambiguously 45 amino acids from the N-terminal end in the E. coli protein and 19 amino acids in the yeast protein. The sequence was identical to the known sequence of hPTH. The sequence analysis indicated that the recombinant PTH was more than 90 percent pure. The recombinant hPTH from E. coli and *Saccharomyces cerevisiae* was fully active in adenylate cyclase assay and also induced hypercalcemia in rats after injection.

On page 9 and bridging page 10, delete the last full paragraph, and replace this paragraph with the following in accordance with 37 C.F.R. §1.121. A marked up version showing changes is attached:

FIG. 13. Purity of purified hPTH (1-84,Q26). Yeast growth medium from yeast strain BJ1991 transformed with the expression plasmids pαUXPTH-Q26 were concentrated and purified by reversed phase HPLC as described in Experimental Protocol. The purity of the recombinant hormone was then analyzed by analytical HPLC (Panel A) and SDS PAGE (Panel B, lane 2). In Panel B the purified hPTH (1-84,Q36) is compared with the wild type hormone purified by two runs on HPLC (lane 3). The molecular weight marker in lane M is the same as in Figure 2. Lane 1 shows a reference PTH produced in *E. coli.*

On page 16 and bridging page 17, delete the last full paragraph, and replace this paragraph with the following in accordance with 37 C.F.R. §1.121. A marked up version showing changes is attached:

hPTH is an easily degraded polypeptide. Already in the parathyroid gland large amounts of carboxyl-terminal PTH fragments are generated.[1/] Structural studies have suggested that hPTH may contain two domains with the easily cleaved region placed in a connecting stalk between these domains.[5/] Not surprisingly therefore, degradation of hPTH has been a major problem when the hormone is expressed in heterologous organisms. In E. coli low expression levels combined with degraded hormone peptides of short half-life were observed.[6-8/] The most successful expression system for hPTH so far is *Saccharomyces*

cerevisiae where the hormone is expressed as a secretory peptide.[9] By that approach we were able to obtain significant amounts of authentic hPTH(1-84) with full biological activity. But even if conditions were found which eliminated proteolytic attacks at some sites in the putative stalk region of the hormone, a significant fraction of the secreted peptides was still cleaved after a pair of basic amino acids found in the hPTH sequence reducing the yield of full length peptide hormone. The cleavage site resembles that recognized by the yscF protease (the KEX2 gene product)).[10,11] We reasoned that the elimination of the putative yscF cleavage in hPTH could lead to a significant gain in the yield of undegraded hPTH secreted from yeast. In the present report we describe the removal of the putative yscF cleavage sites by *in vitro* mutagenesis of the hPTH coding region. When the amino acid at position 26 in hPTH was changed from Lysine (K26) to Glutamine (Q26), the major degradation product hPTH(27-84) previously observed disappeared in the growth medium and the yield of full-length hormone increased 5- to 10-fold. The secreted degradation resistant hPTH(1-84, Q26) had correct size, full immunological reactivity with two different hPTH specific antibodies and correct N-terminal amino acid sequence. Furthermore, the introduced mutation had no effect on the biological activity of the hormone as judged from its action in a hormone-sensitive osteoblast adenylate cyclase assay.

On page 29, delete the second full paragraph, and replace this paragraph with the following in accordance with 37 C.F.R. §1.121. A marked up version showing changes is attached:

The hybridization was carried out at 42 degrees Centigrade for 18 hours in a hybridization solution (6x SSC, 1x Denhart's solution, 20 g/ml tRNA and 0.05% sodium pyrophosphate) supplemented with 32P-labeled DNA probe (Woods supra).

IN THE BRIEF DESCRIPTION OF THE DRAWINGS:

On page 8 and bridging page 9, delete the following Figures, and replace these Figures with the following in accordance with 37 C.F.R. §1.121. A marked up version showing changes is attached:

FIG. 8. Panels a-c illustrate the analysis of expression products by SDS-PAGE and immonoblotting.

Saccharomyces cerevisiae transformed with a PTH cDNA carrying plasmid was grown in liquid culture medium.

The secreted products were concentrated and analyzed on SDS-PAGE. Panel a shown a silver stained gel with molecular size marker (lane S), hPTH standard (lane P), and concentrated yeast growth medium (lane 1). After blotting onto a PVDF membrane, blots were probed with hPTH specific antibodies, one reactive against the aminoterminal part of the hormone (panel b), another reactive against the middle region of the hormone (panel c). Lanes in panel b and c are numbered as in panel a.

FIGS. 9A-9D show the purification of recombinant hPTH medium including: FIG. 9A, a chromatogram of the HPLC purification; in FIG. 9B a chromatogram of the HPLC purification of fractions 32 and 33 from panel 9A (the peak of the recombinant hPTH is indicated in black); FIG. 9C, an HPLC of one microgram standards hPTH (1-84); and 9D, a co-chromatogram of the recombinant PTH from panel 9B and one microgram standard of hPTH.

FIGS. 10A-10G. Construction of PPTH-M13-ΔEA/KQ.

IN THE CLAIMS:

In accordance with 37 C.F.R. § 1.121, please substitute for claim 21 the following rewritten version of the same claim, as amended. The changes are shown explicitly in the attached "Version with Markings to Show Changes Made".

21. (Amended) A process for the production of substantially pure recombinant hPTH, comprising the steps of:

(a) providing a microorganism that is engineered genetically to produce exogenous and intact hPTH(1-84);

(b) expressing said intact hPTH(1-84) within said microorganism; and

(c) purifying said intact hPTH(1-84) so as to produce an intact hPTH(1-84), wherein the intact hPTH(1-84) so produced (1) reacts with antibodies against human PTH in a manner identical to the native hPTH(1-84) hormone, (2) has the molecular weight of the native hPTH(1-84) hormone, and (3) migrates as a single band when subjected to gel electrophoresis.

REMARKS

Status of the Claims

By this amendment, claim 21 is amended. Upon entry of this Amendment, claim 21 will remain pending in the application.

Support for the amendment to claim 21 is found in Example 8 of the present specification (see pages 34-35). Support is also found in the parent application, now U.S. Patent No. 5,010,010 in column 10, lines 22-64.

Because the foregoing amendments do not introduce new matter, entry thereof by the examiner is respectfully requested.

Issues Under Specification

The Examiner objects to the disclosure because the specification contains typographical errors. Applicants have reviewed the disclosure for typographical errors and have corrected the errors.

Issues Under Drawings

The Examiner asserts that the section "Brief Description of the Drawings" should refer to figures with multiple parts. Additionally, the Examiner notes the objections to the Drawings on the PTO-948. Applicants have amended the "Brief Description of the Drawings" section to refer to figures with multiple parts. Additionally, attached herewith are formal drawings.

Claim Rejections - 35 U.S.C. § 112, Second Paragraph

Claim 21 is rejected by the Examiner under 35 U.S.C. § 112, second paragraph, as being indefinite. Applicants respectfully request reconsideration and withdrawal of the rejection.

The Examiner asserts that in claim 21, it is unclear if applicant is claiming hPTH or an analog, such as $hPTH_{Q26}$. Applicants respectfully disagree. Claim 21 states "A process for the production of substantially pure recombinant hPTH..." It is clear from the specification that claim 21 is directed to hPTH(1-84) and is not directed to a hPTH analog. For example, see page 5, line 23 through page 6, line 6 and Example 8.

The Examiner asserts that the term "maximal response" in claim 21 is not clearly defined. Applicants have amended claim 21 by deleting the phrase "which exhibits maximal response which is greater than that which can be achieved by synthetic hPTH." Therefore, the present rejection is moot.

The Examiner also asserts that the phrases "greater than which can be achieved by synthetic hPTH" is a relative term which renders the claim indefinite. Applicants have amended claim 21 by deleting the phrase "which exhibits maximal response which is greater than that which can be achieved by synthetic hPTH." Therefore, the present rejection is moot.

Claim Rejections - 35 U.S.C. § 112, First Paragraph

A. Claim 21 is rejected by the Examiner under 35 U.S.C. § 112, first paragraph, for lack of written description. Applicants respectfully request reconsideration and withdrawal of the rejection.

The Examiner asserts that the term "maximal response" constitutes new matter. Applicants have amended claim 21 by deleting the phrase "which exhibits maximal response which is greater than that which can be achieved by synthetic hPTH." Therefore, the present rejection is moot.

B. Claim 21 is rejected by the Examiner under 35 U.S.C. § 112, first paragraph, for lack of enablement. Applicants respectfully request reconsideration and withdrawal of the rejection.

The Examiner asserts that the specification does disclose a hPTH of the claims that has a maximal response over synthetic hPTH. Applicants have amended claim 21 by deleting the phrase "which exhibits maximal response which is greater than that which can be achieved by synthetic hPTH." Therefore, the present rejection is moot.

Claim Rejections - 35 U.S.C. § 102

Claim 21 is rejected by the Examiner under 35 U.S.C. § 102 as being anticipated by Olstad et al. Applicants respectfully request reconsideration and withdrawal of the rejection.

The Examiner asserts that in making this rejection, Applicants are denied the benefit of the filing dates of prior applications because the prior applications do not describe the presently claimed process. Applicants have amended claim 21 by replacing the phrase "which exhibits maximal response which is greater than that which can be achieved by synthetic hPTH" with --wherein the intact hPTH(1-84) so produced (1) reacts with antibodies against human PTH in a manner identical to the native hPTH(1-84) hormone, (2) has the molecular weight of the native hPTH(1-84) hormone, and (3) migrates as a single band when subjected to gel electrophoresis--. Support for "wherein the intact hPTH(1-84) so produced (1) reacts with antibodies against human PTH in a manner identical to the native hPTH(1-84) hormone, (2) has the molecular weight of the native hPTH(1-84) hormone, and (3) migrates as a single band when subjected to gel electrophoresis" is found in the priority document, now U.S. Patent No. 5,010,010 in column 10, lines 22-64. Therefore, Olstad et al. is not valid prior art against the present application because the priority date of the present application is earlier than the publication date of Olstad et al.

CONCLUSION

As the above-presented amendments and remarks address and overcome all of the rejections presented by the Examiner, withdrawal of the rejections and allowance of the claims are respectfully requested.

If the Examiner has any questions concerning this application, he or she is requested to contact the undersigned.

Respectfully submitted,

Date September 26, 2002

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Should additional fees be necessary in connection with the filing of this paper, or if a petition for extension of time is required for timely acceptance of same, the Commissioner is hereby authorized to charge Deposit Account No. 19-0741 for any such fees; and applicant(s) hereby petition for any needed extension of time.

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

On page 4, the first full paragraph:

In satisfaction of the foregoing objects and advantages, there is provided by the present invention a novel plasmid for insertion in E. coli, containing DNA coding for human preproparathyroid hormone. The plasmid when inserted into E. coli functions to transform the E. coli such that the E. coli then produces multiple copies of the plasmid and thus of the ~~CDNA~~ cDNA coding for human preproparathyroid hormone. The plasmid for human insertion into E. ~~Coli~~ coli of the present invention and thus the transformed E. coli are distinguishable over prior art plasmids and microorganisms, for example as described in Hendy et al., supra, in that the plasmid of the present invention contains a double start codon at the 5' end of the DNA coding for preproparathyroid hormone. The presence of the double start codon may cause ~~a~~ production microorganisms transformed with a plasmid containing the cDNA to produce preproparathyroid hormone at an increased rate and in an improved yield over prior art transformed microorganisms.

On page 6, the second full paragraph:

Employing the promoter and signal sequence of Staphyloccous aureous protein A we have expressed hPTH in ~~*Eacherichis*~~ *Escherichia coli* as a secretory peptide. Immunoreactive PTH was isolated both from growth medium and periplasmic space. We obtained up to 10 mg/1 hPTH as judged by reactivity in radioimmunoassay.

On page 7, the second full paragraph:

HPTH immunoreactive material was concentrated from the growth medium by passage through a S Sepharose Fast flow column and eluted quantitatively. Recombinant hPTH was purified by reverse phase HPLC. The column was eluted with a linear gradient of acetonitrille/trifluoroacetic acid. A major peak (fractions 32 and 33) with the same retention time as standard hPTH(1-84) was resolved into two peaks in a second HPLC ~~urification~~ purification step. The major peak from the 2.HPLC eluted exactly as standard hPTH(1-84)

and co-chromatographed with hPTH(1-84) as one symmetric peak. SDS-PAGE of the peak fraction showed one band co-migrating with hPTH standard suggesting that the recombinant PTH was essentially pure. The recombinant hPTH was subjected to N-terminal amino acid analysis. We were able to determine unambiguously 45 amino acids from the N-terminal end in the E. coli protein and 19 amino acids in the yeast protein. The sequence was identical to the known sequence of hPTH. The sequence analysis indicated that the recombinant PTH was more than 90 percent pure. The recombinant hPTH from E. coli and *Saccharomyces cerevisiae* was fully active in adenylate cyclase assay and also induced hypercalcemia in rats after injection.

On page 9 and bridging page 10, the last full paragraph:

FIG. 13. Purity of purified hPTH (1-84,Q26). Yeast growth medium from yeast strain BJ1991 transformed with the expression plasmids pαUXPTH-Q26 were concentrated and purified by reversed phase HPLC as described in Experimental Protocol. The purity of the recombinant hormone was then analyzed by analytical HPLC (Panel A) and SDS PAGE (Panel B, lane 2). In Panel B the purified hPTH (1-84,Q36) is compared with the wild type hormone purified by two runs on HPLC (lane 3). The molecular weight ~~market~~ marker in lane M is the same as in Figure 2. Lane 1 shows a reference PTH produced in *E. coli.*

On page 16 and bridging page 17, the last full paragraph:

hPTH is an easily degraded polypeptide. Already in the parathyroid gland large amounts of carboxyl-terminal PTH fragments are generated.[1] Structural studies have suggested that hPTH may contain two domains with the easily cleaved region placed in a connecting stalk between these domains.[5] Not surprisingly therefore, degradation of hPTH has been a major problem when the hormone is expressed in heterologous organisms. In E. coli low expression levels combined with degraded hormone peptides of short half-life were observed.[6-8] The most successful expression system for hPTH so far is *Saccharomyces cerevisiae* where the hormone is expressed as a secretory peptide.[9] By that approach we were able to obtain significant amounts of authentic hPTH(1-84) with full biological activity. But even if conditions were found which eliminated proteolytic attacks at some sites in the

putative stalk region of the hormone, a significant fraction of the secreted peptides was still cleaved after a pair of basic amino acids found in the hPTH sequence reducing the yield of full length peptide hormone. The cleavage site resembles that recognized by the yscF protease (the KEX2 gene product)).[10,11] We reasoned that the elimination of the putative yscF cleavage in hPTH could lead to a significant gain in the yield of undegraded hPTH secreted from yeast. In the present report we describe the removal of the putative yscF cleavage sites by *in vitro* mutagenesis of the hPTH coding region. When the amino acid at position 26 in hPTH was changed from Lysine (K26) to Glutamine (Q26), the major degradation product hPTH(27-84) previously observed disappeared in the growth medium and the yield of full-length hormone increased 5- to 10-fold. The secreted degradation resistant ~~hPTH)1-84, Q26)~~ hPTH(1-84, Q26) had correct size, full immunological reactivity with two different hPTH specific antibodies and correct N-terminal amino acid sequence. Furthermore, the introduced mutation had no effect on the biological activity of the hormone as judged from its action in a hormone-sensitive osteoblast adenylate cyclase assay.

On page 29, the second full paragraph:

The hybridization was carried out at 42 degrees Centigrade for 18 hours in a hybridization solution (6x SSC, 1x Denhart's solution, 20 g/ml tRNA and 0.05% sodium ~~pyrophophate~~ pyrophosphate) supplemented with 32P-labeled DNA probe (Woods supra).

IN THE BRIEF DESCRIPTION OF THE DRAWINGS:

On page 8 and bridging page 9, the following figures:

FIG. 8. ~~Analysis of expression products by SDS-PAGE and immonoblotting.~~ Panels a-c illustrate the analysis of expression products by SDS-PAGE and immonoblotting.

Saccharomyces cerevisiae transformed with a PTH cDNA carrying plasmid was grown in liquid culture medium.
The secreted products were concentrated and analyzed on SDS-PAGE. Panel a shown a silver stained gel with molecular size marker (lane S), hPTH standard (lane P), and concentrated

yeast growth medium (lane 1). After blotting onto a PVDF membrane, blots were probed with hPTH specific antibodies, one reactive against the aminoterminal part of the hormone (panel b), another reactive against the middle region of the hormone (panel c). Lanes in panel b and c are numbered as in panel a.

~~Figure 9. Purification of recombinant hPTH from the growth medium.~~

~~A: Chromomatogram of the 1.HPLC purification~~

~~B: Chromatogram of the 2.HPLC purification of fractions 32 and 33 from panel A. The peak of the recombinant hPTH is indicated by black.~~

~~C: 2.HPLC run of 1 ug standard hPTH(1-84)~~

~~D: Co-chromatography of the recombinant PTH pack from panel B and 1 ug of standard hPTH (1-84)~~

~~E: Silver staining of SDS-PAGE of the proteins in the hPTH pack~~

~~1: recombinant hPTH, 1 ug~~

~~2: hPTH(1-84) (o), 3ug (Note HMW Impurities)~~ FIGS. 9A-9D show the purification of recombinant hPTH medium including: FIG. 9A, a chromatogram of the HPLC purification; in FIG. 9B a chromatogram of the HPLC purification of fractions 32 and 33 from panel 9A (the peak of the recombinant hPTH is indicated in black); FIG. 9C, an HPLC of one microgram standards hPTH (1-84); and 9D, a co-chromatogram of the recombinant PTH from panel 9B and one microgram standard of hPTH.

~~Figure 10. Construction of PPTH-M13-ΔEA/KQ.~~ FIGS. 10A-10G. Construction of PPTH-M13-ΔEA/KQ.

IN THE CLAIMS:

21. (Amended) A process for the production of substantially pure recombinant hPTH, comprising the steps of:

(a) providing a microorganism that is engineered genetically to produce exogenous and intact hPTH(1-84);

(b) expressing said intact hPTH(1-84) within said microorganism; and

(c) purifying said intact hPTH(1-84) so as to produce an intact hPTH(1-84), wherein the intact hPTH(1-84) so produced (1) reacts with antibodies against human PTH in a manner identical to the native hPTH(1-84) hormone, (2) has the molecular weight of the native hPTH(1-84) hormone, and (3) migrates as a single band when subjected to gel electrophoresis [which exhibits maximal response which is greater than that which can be achieved by synthetic hPTH].